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Preferential PCR Amplification of Parasitic Protistan Small Subunit rDNA from Metazoan Tissues

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ABSTRACT. A "universal non-metazoan" polymerase chain reaction (UNonMet-PCR) that selectively amplifies a segment of non-metazoan Small Subunit (SSU) rDNA gene was validated. The primers used were: 18S-EUK581-F (5'-GTGCCAGCAGCCGCG-3') and 18S-EUK1134-R (5'-TTTAAGTTTCAGCCTTGCG-3') with specificity provided by the 19-base reverse primer. Its target site is highly conserved across the Archaea, Bacteria, and eukaryotes (including fungi), but not most Metazoa (except Porifera, Ctenophora, and Myxozoa) which have mismatches at bases 14 and 19 resulting in poor or failed amplification. During validation, UNonMet-PCR amplified SSU rDNA gene fragments from all assayed protists (n = 16 from 7 higher taxa, including two species of marine phytoplankton) and Fungi (n = 3) but amplified very poorly or not at all most assayed Metazoa (n = 13 from 8 higher taxa). When a non-metazoan parasite was present in a metazoan host, the parasite DNA was preferentially amplified. For example, DNA from the parasite *Trypanosoma danilewskyi* was preferentially amplified in mixtures containing up to 1,000× more goldfish *Carassius auratus* (host) DNA. Also, the weak amplification of uninfected host (*Chionoecetes tanneri*) SSU rDNA did not occur in the presence of a natural infection with a parasite (*Hematodinium* sp.). Only *Hematodinium* sp. SSU rDNA was amplified in samples from infected *C. tanneri*. This UNonMet-PCR is a powerful tool for amplifying SSU rDNA from non-metazoan pathogens or symbionts that have not been isolated from metazoan hosts.

Key Words. Molecular tool, preferential amplification of parasite DNA, SSU rDNA fragment.

THE polymerase chain reaction (PCR) is proving to be a significant research and diagnostic tool for the detection of protistan pathogens in commercially important aquatic organisms (e.g. Carnegie et al. 2000; Cunningham 2002; Stokes, Siddall, and Burreson 1995). Its specificity and often exquisite sensitivity make it a powerful complement to conventional histopathological techniques already used for parasite detection. However, the crucial first step to the development of specific molecular assays is the characterisation of a diagnostic gene such as that coding for SSU rDNA (Hillis and Dixon 1991) and this step is often difficult. In some cases, the characterisation of parasite genes has failed because pathogen DNA could not be separated from host DNA. The application of "universal" eukaryotic PCR primers may amplify both pathogen and host DNA, but because host DNA often predominates in bulk mixtures, it is preferentially amplified to the exclusion of parasite DNA. Alternatively, amplification of parasite genes may fail because the target sequences for "universal" eukaryotic PCR primers are divergent in the genome of the pathogen. Pure isolates of many pathogens are unavailable as axenic cultures, further confounding the ability to characterise these organisms. Thus, molecular analyses, with all the associated advantages including the development of sensitive and specific diagnostic assays, are not possible or proceed slowly.

This dilemma was exemplified by *Mikrocytos mackini*, a protistan parasite of unknown taxonomic affiliation, which causes Denman Island disease in wild and aquacultured oysters (*Crassostrea gigas*, *Crassostrea virginica*, *Ostrea edulis*, and *Ostrea conchaphila*) (Bower, Hervio, and Meyer 1997; Farley, Wolf, and Elston 1988; Hervio, Bower, and Meyer 1996; Hine et al. 2001; Quayle 1961). After various unsuccessful attempts to identify *M. mackini* DNA using universal eukaryotic PCR primers, a set of PCR primers designed not to amplify host DNA generated a 544-base pair (bp) 18S rDNA fragment from *M. mackini*-infected oysters and enriched *M. mackini* isolates, but not from uninfected control oysters. The gene fragment with subsequent elongation was confirmed to be *M. mackini* 18S rDNA by fluorescent in situ hybridization (Carnegie et al. 2003). Both forward and reverse primers (18S-EUK581-F: 5'-GTGCCAGCAGCCGCG-3' and 18S-EUK-1134-R: 5'-TTTA AGTTTCAGCCTTGCG-3', hereafter referred to as the UNonMet-PCR), were designed to preferentially amplify parasite DNA. These primers targeted regions highly conserved across all sequences in an alignment of eukaryotes, Archaea and Bacteria. The reverse primer, however, was mismatched to the target sequence in metazoan 18S rDNA at the 14th and 19th (final) positions (Fig. 1). It was therefore expected to amplify metazoan rDNA inefficiently, if at all. The success achieved by Carnegie et al. (2003) indicated the potential application of this new UNonMet-PCR to identify SSU rDNA of pathogens in metazoa.

The purpose of the present study was to identify the wider application of the UNonMet-PCR. In addition to comparing the primer target sites with SSU rDNA sequences available in GenBank, the capability of the primers to amplify a segment of the SSU rDNA gene from 32 representative species of 16 higher eukaryotic taxa was tested. In addition, the suitability of the UNonMet-PCR to amplify bacterial DNA was informally assessed.

MATERIALS AND METHODS

General approach. Gene segments of the predicted length resulting from the UNonMet-PCR of test samples were sequenced and the sequences compared with those available in GenBank to determine the homology of the amplified segment. The quality of the DNA in metazoan samples that produced no or poor products (i.e. that could not be sequenced) with the UNonMet-PCR was verified using known specific or universal primers. The limitation of the UNonMet-PCR was tested on separate mixtures of DNA from two haemoflagellate parasites with DNA from a cell line derived from their respective fish hosts (*Trypanosoma danilewskyi* with the *Cyprinus carpio* EPC cell line and *Cryptobia salmositica* with the *Oncorhynchus tshawytscha* CHSE 214 cell line) and on serial dilutions of *T. danilewskyi* DNA with host (*Carassius auratus*) DNA. In addition, the relevance of the proposed UNonMet-PCR was tested on natural infections of *Hematodinium* sp. in *Chionocetes tanneri* and results compared with histological examination and a *Hematodinium* genus-specific PCR.

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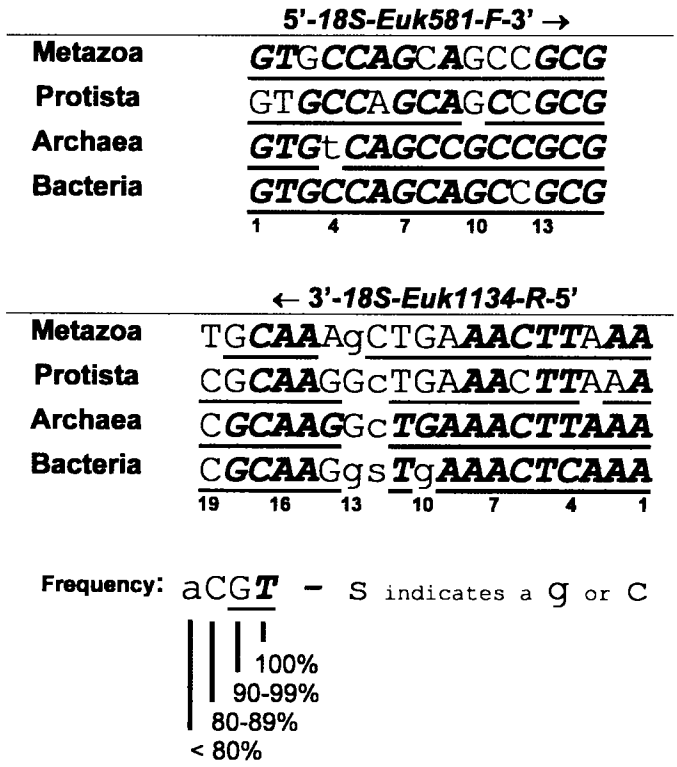

Fig. 1. Conservation of 18S–581-F (top) and 18S–1134-R (bottom) SSU rDNA target regions in the Metazoa, protists, Archaea, and Bacteria used in the UNonMet-PCR. For clarity, sense sequences are shown for both target sites. Arrows indicate the directionality of the primers and numbering denotes the position in the primers. Frequencies indicate the estimated level of conservation at each target position among the 294 metazoan, protistan, archaeal, and bacterial species that were aligned. In the 19th position of the 18S-Euk1134-R target site, for example, the metazoan "T"—not bold or underlined—indicates that 80–89% of metazoan species in the alignment displayed a T at this position.

Collection and isolation of DNA. DNA was obtained from a total of 32 species of eukaryotes represented by one isolate in most cases (Table 1). These isolates represented 16 higher taxa in the superkingdom Eukaryota according to taxonomic information obtained from the GenBank database of the National Center for Biotechnology Information (July 2003). DNA was purified from the organisms using the DNeasy™ Tissue Kit (Qiagen Operon Technologies, Inc., Mississauga, Ontario, Canada) following the instructions provided for isolation of DNA from animal tissue, cultured cells, whole nucleated blood or yeast depending on the nature of the sample (Table 1). The concentration of isolated DNA was determined using a Genequant pro RNA/DNA Calculator (Biochrom Ltd., Cambridge, U.K.) and all samples were diluted to between 4 and 40 ng/μl in Tris-EDTA (TE) buffer (pH 7.8) for use as a PCR template. All primers used during this study were obtained from either Qiagen Operon Technologies, Inc. or Invitrogen Canada Inc. (Burlington, Ontario, Canada).

In addition, DNA was obtained from cultures of two Gram-negative (*Aeromonas salmonicida* and *Escherichia coli*) and two Gram-positive (*Renibacterium* Dr143, *Nocardia crassostreae*) bacteria. Each sample was assayed with the UNonMet-PCR at various annealing temperatures between 47 °C and 66 °C. Also, DNA integrity of the bacteria was confirmed using the 16S rDNA universal primers 29–47 forward and Univ1492r described by Rainey et al. (1992) and Jackson et al. (2001), respectively.

Assaying the specificity of the UNonMet-PCR. The PCR reaction mixture contained PCR buffer at 1× concentration; $MgCl_2$ at 2.5 mM; nucleotides at 0.2 mM; primers 581F and 1134R at 0.05 μM; Platinum *Taq* DNA polymerase at 0.05 units/μl (all reagents purchased from Invitrogen Canada Inc.); and 6–60 ng of template DNA. The reaction vol. was 15 μl. The temperature profile included an initial denaturation step at 94 °C for 10 min, followed by 40 cycles of amplification (94 °C for 1 min, annealing temperature (optimised for each reaction; see below) for 1 min, and extension at 72 °C for 1 min) and a final extension at 72 °C for 10 min on a PTC200 thermocycler (MJ Research, Waltham, Massachusetts, USA). The optimal annealing temperature (Table 2) was determined by running a PCR with an annealing temperature gradient of 49–62 °C. The products were electrophoresed on a 1.5% agarose (in 1× Tris-borate EDTA buffer) gel containing 0.1 μg/ml ethidium bromide and were visualised using UV light.

The DNA from *Mikrocytos mackini*, *Alexandrium catenella* (a photosynthetic dinoflagellate) or *Saccharomyces cerevisiae* (baker's yeast) was used as a positive control in the PCR. The negative control was PCR-grade water used in place of template DNA.

Identification of UNonMet-PCR products. The UNonMet-PCR reactions immediately preceding sequencing were performed in larger 50-μl vol. with 5 μl of template DNA. If amplification generated a single band of around the predicted size (based on the alignment of the PCR primers with the target or a related sequence in Genbank), the PCR product was sequenced directly. If multiple distinct bands were produced, the band of interest was cut from the gel, immersed in 0.2 ml of TE buffer and subjected to three freeze (−20 °C) and thaw (room temperature) cycles. The resulting elution was re-amplified and sequenced. If the multiple bands could not be adequately separated electrophoretically, the components of the mixed amplification product were isolated by PCR cloning using a TOPO TA Cloning Kit (Invitrogen Canada Inc.) and then sequenced.

If amplification using UNonMet-PCR failed, the quality of the DNA in the sample was assayed by PCR with specific primers (for *Oncorhynchus tshawytscha*, *Salmo salar*, *Caenorhabditis elegans*, *Loma salmonae*, and *Kudoa thyrsites*) and protocols as required. Samples from species for which specific primers were not available (*Carassius auratus*, *Chionoecetes tanneri*, *Cancer magister*, *Rhopalura ophiocomae*, and *Crassostrea gigas*) were assayed using universal primers (18E and 18G) described by Hillis and Dixon (1991). With these primers, the above PCR procedure was used except that the final nucleotide concentration was increased to 0.25 mM and the annealing and extension times for each cycle were increased to 90s from 60s.

Bidirectional sequences of the isolated UNonMet-PCR products were determined using BigDye Terminator (Invitrogen Canada Inc.) or BigDye Primer (Invitrogen Canada Inc.) sequencing reactions with the 581F/1134R-primer set and subsequent electrophoresis on an ABI 377 Automated DNA Sequencer (Applied Biosystems, Foster City, California, USA). The percent homology of each product was determined using a Genbank BLAST search (Altschul et al. 1997) for a corresponding organism.

Analysis of bands produced by metazoa. In order to analyse the UNonMet-PCR amplification of DNA from metazoa, DNA samples from 40 *Cancer magister* and 32 *Crassostrea gigas* were tested using the procedure described above. The

Table 1. Taxonomic affiliation (used in GenBank), material received for analysis, source and DNA extraction protocol (if applicable) of organisms tested with the UNonMet-PCR.

Species (number of sampled examined)	Taxonomic affiliation	Materials	Source	DNeasy™ (Qiagen) protocol used
EUKARYOTES exclusive of FUNGI and METOZOA				
Alexandrium catenella (1)	Alveolata	live, axenic culture	J.N.C. Whyte, Pacific Biological Station, Canada	animal tissue
Hematodinium sp. (13)	Alveolata	alcohol preserved sample	S. Bower, Pacific Biological Station, Canada	animal tissue
Perkinsus marinus—C14-13, clone of ACC50738 (1)	Alveolata	alcohol preserved culture	D. Bushek, Virginia Institute of Marine Science, USA	animal tissue
Rhodomonas sp. (1)	Cryptophyta	live, axenic culture	J.N.C. Whyte, Pacific Biological Station, Canada	animal tissue
Cryptobia salmositica—T4 vaccine (1)	Euglenozoa	alcohol preserved culture	P.T.K. Woo, University of Guelph, Canada	animal tissue
Trypanosoma danilewskyi (1)	Euglenozoa	alcohol preserved culture	M. Belosevic, University of Alberta, Canada	animal tissue
Chrysochromulina sp. (1)	Haptophyceae	live, axenic culture	J.N.C. Whyte, Pacific Biological Station, Canada	animal tissue
Isochrysis sp.—CCMP 1324 (1)	Haptophyceae	live, axenic culture	J.N.C. Whyte, Pacific Biological Station, Canada	animal tissue
Pavlova lutheri—CCMP 1235 (1)	Haptophyceae	live, axenic culture	J.N.C. Whyte, Pacific Biological Station, Canada	animal tissue
Bangia fuscopurpurea (1)	Rhodophyta	live, axenic culture	K. Muller, University of Waterloo, Canada	animal tissue
Colaconema caespitosum—GWSC 3582 (1)	Rhodophyta	DNA	G. Saunders, University of New Brunswick, Canada	not applicable
Chaetoceros gracilis (1)	stramenopiles	live, axenic culture	J.N.C. Whyte, Pacific Biological Station, Canada	animal tissue
Heterosigma akashiwo (1)	stramenopiles	live, axenic culture	J.N.C. Whyte, Pacific Biological Station, Canada	animal tissue
Thalassiosira pseudonana (1)	stramenopiles	live, axenic culture	J.N.C. Whyte, Pacific Biological Station, Canada	animal tissue
Arabidopsis thaliana (1)	Viridiplantae	DNA	B. Yu, Pacific Forestry Centre, Canada	not applicable
Tetraselmis suecica (1)	Viridiplantae	live, axenic culture	J.N.C. Whyte, Pacific Biological Station, Canada	animal tissue
		FUNGI		
Saccharomyces cerevisiae (1)	Fungi	live, axenic culture	commercial product, Fermipan®, Lallemand Inc. USA	yeast
Cronartium ribicola (1)	Fungi	DNA	B. Yu, Pacific Forestry Centre, Canada	not applicable
Loma salmonae (1)	Fungi	alcohol preserved spores	S. Jones, Pacific Biological Station, Canada	animal tissue
		METAZOA		
Pleurobrachia bachei (1)	Ctenophora	fresh tissue	B. Goh, West Vancouver Labs, Canada	animal tissue
Suberites ficus (1)	Porifera	DNA	A. Collins, Institut fur Tierokologie und Zellbiologie, Germany	not applicable
Homarus americanus (2)	Arthropoda	alcohol preserved tissue	S. Bower, Pacific Biological Station, Canada	animal tissue
Cancer magister (40)	Arthropoda	alcohol preserved tissue and haemolymph	S. Bower, Pacific Biological Station, Canada	animal tissue or whole nucleated blood
Chionoecetes tanneri (67)	Arthropoda	alcohol preserved haemolymph	S. Bower, Pacific Biological Station, Canada	whole nucleated blood
Cyprinus carpio—EPC cell line (1)	Chordata	live culture	J. Robinson, Animal Health Centre, Canada	cultured cells
Carassius auratus (3)	Chordata	fresh tissue	local pet store	animal tissue
Oncorhynchus tshawytscha—CHSE 214 cell line (1)	Chordata	live culture	J. Robinson, Animal Health Centre, Canada	cultured cells
Oncorhynchus tshawytscha (2)	Chordata	DNA	K.M. Miller, Pacific Biological Station, Canada	not applicable
Salmo salar (1)	Chordata	DNA	K.M. Miller, Pacific Biological Station, Canada	not applicable
Rhopalura ophiocomae (1)	Mesozoa	alcohol preserved tissue	C. Adema, University of New Mexico, USA	animal tissue
Crassostrea gigas (32)	Mollusca	alcohol preserved tissue	S. Bower, Pacific Biological Station, Canada	animal tissue
Kudoa thyrsites (1)	Myxozoa	alcohol preserved spores	S. Jones, Pacific Biological Station, Canada	animal tissue
Caenorhabditis elegans—N2 genomic (1)	Nematoda	DNA	D. Baillie, Simon Fraser University, Canada	not applicable

Table 2. The number of bands (in a 1.5% agarose gel stained with ethidium bromide) resulting from UNonMet-PCR products produced at optimum annealing temperatures and the percent homology obtained by BLAST analysis of sequenced PCR product with sequences given in GenBank. For source of species tested see Table 1.

Species (number of isolates sequenced)	Number of bands produced	Optimal annealing temperature (°C)	% Homology with sequence in GenBank (accession number)
EUKARYOTES exclusive of FUNGI and METOZOA			
Alexandrium catenella (1)	one	57	98 with *A. catenella* (AB088335), 98 with *Alexandrium tamarense* (AF022191)
Hematodinium sp. (2)	one	55	97 (AF421)
Perkinsus marinus (1)	one	55	98 (X75762)
Rhodomonas sp. (1)	three[a]	56	79 with *Rhodomonas* sp. (AJ420694), 97 with *Tetraselmis striata* (X70802)
Cryptobia salmositica (1)	one	49	95 (AF080225)
Trypanosoma denilewskyi (1)[c]	one	50	95 with *Trypanosoma cobitis* (AJ009143)
Chrysochromulina sp. (1)	two[b]	57	100 with *Chrysochromulina hirta* (AJ246272)
Isochrysis sp. (CCMP 1324) (1)	two[b]	49	99 with *Isochrysis galbana* (AJ246266)
Pavlova lutheri (CCMP 1235) (1)	one	49	100 (AB058362)
Bangio fuscopurpurea (1)	three[a]	62	97 (AF169336)
Colaconema caespitosum (1)	one	49	99 (AF079787)
Chaetoceros gracilis (1)	one	49	94 with *Chaetoceros* sp. (AF145226 and X85390)
Heterosigma akashiwo (1)	one	49	99 (AB001287)
Thalassiosira pseudonana (1)	one	49	99 (AF374481)
Arabidopsis thaliana (1)	one	49	100 (AC006837)
Tetraselmis suecica	two[b]	57	98 (U41900)
FUNGI			
Saccharomyces cerevisiae (1)	one	61	98 (Z75578)
Conrartium ribicola (1)	two[a]	56	75 with *C. ribicola* (M94338), 96 with *Pinus elliottii* (D38245)
Loma salmonae (1)[c]	two[a,b]	56	95 with *Candida coipomoensis* (AB013561)[a], sequence unreadable[b]
METAZOA			
Pleurobrachia bachei (1)	one	55	100 (AF293677)
Suberites ficus (1)	one	61	100 (AF100947)
Homarus americanus (2)	one	57	96 (AF235971)
Cancer magister (2)[c]	variable[a]	49	97 with *Hepatus epheliticus* (AF436004)
Chionoecetes tanneri (1)[c]	variable[b]	56	sequence unreadable
Cyprinus carpio (EPC) (0)	three to ten	49–62	not sequenced
Carassius auratus (1)[c]	one[d]	49	sequence unreadable
Oncorhynchus tshawytscha (CHSE-214) (0)	three to ten	49–62	not sequenced
Oncorhynchus tshawytscha (1)	one	51	sequence unreadable
Salmo salar (0)	none	49–62	not applicable
Rhopalura ophiocomae (0)	none	49–62	not applicable
Crassostrea gigas (1)	variable[b]	49	42 with *C. gigas* (AB064942), 90 with *Ephelota* sp. (AF326357) but containing large gaps
Kudoa thyrsites (1)[c]	two[a,b]	56	99 with *Festuca rubra* (AF168844)[a] sequence unreadable[b]
Caenorhabditis elegans (1)	none	51–62	not applicable

[a] Amplicon of interest isolated for DNA sequencing using a TOPO TA Cloning Kit (Invitrogen Canada Inc.).
[b] Amplicon of interest isolated for DNA sequencing using gel extraction procedure described in Materials and Methods.
[c] None or only partial SSU rDNA sequence data available in GenBank for species tested.
[d] Band was about 200 bp in length.

600-bp band produced in two crabs and one oyster were sequenced as above.

Detection of non-metazoan DNA in a mixture. *Trypanosoma danilewskyi* DNA was serially diluted from 10 ng/μl to 10^{-10} ng/μl and each dilution was mixed with 10 ng/μl *C. carpio* DNA (isolated from an EPC cell line). The reverse dilution series was also made by diluting *C. carpio* DNA from 500 ng/μl to 10^{-10} ng/μl and mixing these dilutions with 10ng/μl *T. danilewskyi* DNA. Each mixture was then assayed in a PCR reaction using the UNonMet-PCR as described above. The experiment was repeated using *C. salmositica* DNA and *O. tshawytscha* DNA (isolated from the CHSE-214 cell line) except the *O. tshawytscha* DNA concentration ranged from 75 ng/μl to 10^{-10} ng/μl. These procedures were performed three times to confirm detection sensitivity. In addition, each of the above serial dilutions of *T. danilewskyi* DNA was mixed with 10 ng/μl *Carassius auratus* DNA isolated directly from the muscle of a goldfish. To confirm and refine the minimum amount of *T. danilewskyi* DNA detectable in the mixture, the procedure was repeated eight times using narrower dilution series (10^{-2} ng/μl to 10^{-4} ng/μl).

Application of assay to natural parasitic infections. The utility of the UNonMet-PCR for amplifying parasite DNA in infected animals was tested on natural parasitic dinoflagellate (*Hematodinium* sp.) infections in Tanner crabs (*Chionoecetes tanneri*). Tissue samples were obtained from 67 selected *C. tanneri* collected about 10 miles off the west coast of Vancouver Island, British Columbia, Canada during the months of Jan-

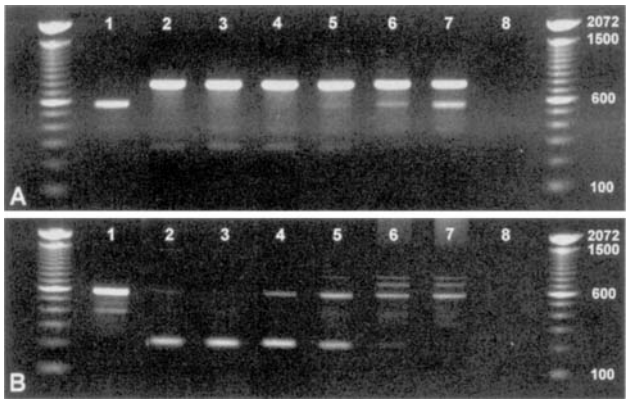

Fig. 2. Effects of annealing temperature on the amplification of SSU rDNA by UNonMet-PCR as visualised with ultraviolet light in agarose gel (1.5%) stained with ethidium bromide. For both gels, the annealing temperatures of each PCR increased from lanes 2 to 7 (49.4, 52.7, 55.6, 57.8, 59.4, 60.6 °C respectively), while the annealing temperature for lanes 1 and 8 were 49 °C. In both gels, the first and last lanes are molecular markers, lane 1 contains DNA from the positive control (*Alexandrium catenella*), and lane 8 contains the negative control (ddH_20). In gel A, lanes 2 to 7 contain DNA from *Cryptobia salmositica* (T-4 Vaccine, 10 ng in a 15-µl PCR), and in gel B, lanes 2 to 7 contain DNA from *Carassius auratus* (10 ng in a 15-µl PCR).

uary, March, and April 2001. Haemolymph samples were preserved in 95% ethanol and samples of the hepatopancreas, gills, heart and/or muscle tissue from each crab were fixed in Davidson's solution. For DNA analysis, cells in the haemolymph samples were concentrated by centrifugation and processed using the animal tissue protocol of the DNeasy™ Tissue Kit. The DNA in each sample was separately analysed using the UNonMet-PCR (predicted product size: 600 bp) and the genus-specific primers HEMAT 18SF (GAACCGAACCAAGCTCT GCTTGGCC) and HEMAT 18SR (CCAAAGGGTGCACCG ATCGCTTCAA) (predicted product size: 450 bp). The PCR conditions were as indicated above except for assays using the HEMAT 18S primers in which the initial 94 °C hold was shorter (2 min), the annealing temperature was higher (60 °C), the times for melting, annealing and extension were shorter (0.5 min for each) and the final concentration of $MgCl_2$ was lower (1.5 mM). For histopathological analysis, tissues preserved in Davidson's solution were processed and examined microscopically according to standard procedures (Howard and Smith 1983). Results obtained using the UNonMet-PCR were compared with those from histopathological examinations and PCR assays using genus-specific primers.

RESULTS

Amplification by UNonMet-PCR. The UNonMet-PCR generated an amplification product of predicted size (550–850 bp) from all eukaryotic non-metazoa at all annealing temperatures (49–62 °C). However, samples from some non-metazoa produced one or two additional faint bands (ranging from about 400–700 bp) that were different from the predicted size for that species (Table 2). These faint bands were not analysed. The metazoa (exclusive of the fish cell lines) inconsistently produced several bands ranging in size from 400–850 bp at lower annealing temperatures and occasional faint bands at higher temperatures (Table 2). Many bands ranging in size from about 200–2,000 bp were amplified from the two fish cell lines (EPC and CHSE 214). As the annealing temperature was increased, the banding patterns for the non-metazoa tended to remain consistent (Fig. 2A) but those derived from metazoan samples faded and appeared inconsistently at various temperatures (Fig. 2B). The optimum annealing temperature for the various organisms tested (Table 2) was determined by the presence of the least number of secondary bands and the strongest band in the predicted size range on the gel for each species.

The DNA from all four species of bacteria assayed with the UNonMet-PCR produced two strong and several weak bands over a range of annealing temperatures. One strong band larger than 2,000 bp was produced at all annealing temperatures (47–66 °C) and the other strong band of about 600 bp was produced at lower annealing temperatures (below 58 °C). The band of expected size (about 400 bp) was one of the weaker bands produced at lower annealing temperatures, and none of the bands was further analysed. Nevertheless, the integrity of the bacterial DNA was confirmed by production of expected bands of slightly more than 1,500 base pairs in size for each isolate using the16S rDNA 29–47 forward and Univ1492r primer set.

Sequencing of UNonMet-PCR amplicons from non-metazoa. Sequencing of PCR products of predicted size and subsequent BLAST analysis confirmed that the UNonMet-PCR had amplified a segment of the SSU rDNA with 94% to 100% homology to the sequence for conspecific or closely related eukaryotic species in GenBank in most cases (Table 2). The sequence from the sample received as *Rhodomonas* sp. matched more closely with that of *Tetraselmis striata*. The supplier of the sample indicated that in the 17 yr since the sample had been collected it had probably become contaminated with *T. striata* which was commonly cultured in the laboratory (Whyte, J. N. C., pers. commun.). The samples of *Isochrysis* sp. and *Chrysochromulina* sp. that were tested aligned most closely with *I. galbana* (99% homology), and *C. hirta* (100% homology), respectively. There were no published SSU rDNA sequences for *Trypanosoma danilewskyi* and *Chaetoceros gracilis*. The sequenced product for *T. danilewskyi* aligned most closely (95% homology) with *Trypanosoma cobitis*, another parasite of fish; *C. gracilis* aligned most closely (94% homology) with two unidentified *Chaetoceros* sp. isolates.

Of the three fungi assayed, only one (*Saccharomyces cerevisiae*) produced a clear band that was confirmed by sequencing to be the predicted amplification product (Table 2). Unexpected sequencing results from the other two species were attributable to contamination in the original samples (see discussion). The sequence of *Loma salmonae* did not match that of *L. salmonae* in GenBank but was 95% homologous to the yeast *Candida coipomoensis*. Also, the sequence of *Cronartium ribicola* (a fungal parasite of trees) was only 75% homologous to the *C. ribicola* sequence in GenBank and the sequence most closely resembled the SSU rDNA gene of *Pinus elliottii* (96% homology, no SSU rDNA sequence available for the host tree, *Pinus monticola*, in GenBank).

Analysis of bands produced by metazoa. Only 3 of the 13 metazoa assayed, a Ctenophora, a Porifera and one Arthropoda, produced a clear band that when sequenced resulted in at least 96% homology to the sequence of the organism assayed (Table 2). The other 10 species did not produce amplicons (with the UNonMet-PCR) that could be sequenced or gave aberrant results. The integrity of the DNA in these samples (except of the fish cell lines) was verified using other primers (Table 3). Specific primers applied to five of the samples resulted in PCR amplicons of expected size. The other five samples assayed with the universal SSU rDNA primers 18E and 18G described by Hillis and Dixon (1991) also gave good results. Samples from two species resulted in sequences greater than 96% homology to sequences for respective species in GenBank. There were no published SSU rDNA sequences for the other three metazoans (*Cancer magister*, *Chionoecetes tanneri* and *Car-*

Table 3. Confirmation of DNA integrity in samples of a fungus (*Loma salmonae*) and the metazoa that did not produce amplicons that could be sequenced or gave aberrant results with the UNonMet-PCR.

Species	Primers used	Optimal annealing temperature (°C)	Amplicon size (bp)	% Homology with sequence in GenBank (accession number)
Loma salmonae	Species specific LS1 and LS2 (Docker et al. 1997)	56	272	Not sequenced[a]
Cancer magister	Universal 18E and 18G (Hillis and Dixon 1991)	60	1600	98 with *Raninoides louisianensis* (AF436005)
Chionoecetes tanneri	Universal 18E and 18G (Hillis and Dixon 1991)	60	1600	99 with *Helice tridens tientsinensis* (HTTR18SRR)
Carassius auratus	Universal 18E and 18G (Hillis and Dixon 1991)	49	800	99 with *Cyprinus carpio* (AF133089)
Oncorhynchus tshawytscha	Salmon specific A1Rc1 and A1ef (K.M. Miller pers. commun.)	51	250	Not sequenced[a]
Salmo salar	Salmon specific A1Rc1 and A1ef (K.M. Miller pers. commun.)	51	250	Not sequenced[a]
Rhopalura ophiocomae	Universal 18E and 18G (Hillis and Dixon 1991)	60	1600	97.5 (U58361)
Crassostrea gigas	Universal 18E and 18G (Hillis and Dixon 1991)	60	1600	96.5 (AB064942)
Kudoa thyrsites	Species specific Kt18S-3F and Kud-3R (Jones, Goh, and Prosperi-Porta 2003)	56	531	Not sequenced[a]
Caenorhabditis elegans	Species specific C50F4.11A forward and C50F4.11B reverse (Kitagawa and Rose 1999)	49	659	Not sequenced[a]

[a] PCR amplicon produced band of expected size on gel.

assius auratus). However, the resulting sequences were most similar to that of the crab *Raninoides louisianensis*, the crab *Helice tridens tientsinensis* and the carp *Cyprinus carpio*, respectively.

The inconsistent amplification of metazoan DNA with the UNonMet-PCR was investigated in detail with two invertebrates, *Crassostrea gigas* (Pacific oyster) and *Cancer magister* (Dungeness crab). Within the range of annealing temperatures tested (49–62 °C) various sized bands were inconsistently produced from a sample of each species. Thus, many individuals of each species were tested using an annealing temperature of 49 °C. Of the 32 samples of *C. gigas* tested, five failed to show any amplification of DNA while the other 27 samples produced several banding patterns. Eight of these produced a faint band of approximately 600 bp, 12 produced strong bands of about 550 bp, four produced two bands of both sizes (of which the 550-bp band was stronger), and three produced multiple bands ranging from 400–600 bp. One of the 12 samples that produced a single strong band (550 bp) was sequenced and the sequence aligned most closely with a ciliate (Suctoria) (Table 2). The quality of the DNA in this sample was verified using primers 18E and 18G of Hillis and Dixon (1991). The resulting sequence was 96.5% homologous to the sequence for *C. gigas* in GenBank.

Of the 40 samples of *C. magister* tested, nine failed to show any amplification of DNA while the other 31 samples produced several banding patterns. Nine of these produced one strong band of approximately 600 bp. The other 22 produced multiple bands (three to five) ranging in size from 300–700 bp with the strongest band being 600 bp. One of the nine samples that gave a single strong 600-bp band was sequenced, and the 600-bp sequence was found to be 97% homologous to the SSU rDNA gene of *Hepatus epheliticus*, the calico box crab (there was no sequence for *C. magister* in GenBank). A product obtained from the same sample using primers 18E and 18G of Hillis and Dixon (1991) had 98% homology to another species of crab *Raninoides louisianensis*. Also, 189 bps of the 5′ end of this amplicon overlapped with the 3′ end of the amplicon produced using UNonMet-PCR (99% homology within the overlapping segment).

Detection of non-metazoan DNA in a mixture. A parasite-specific PCR product was produced from a mixture of *T. danilewskyi* DNA and EPC cell line DNA when the concentration of EPC DNA was not greater than 250× that of *T. danilewskyi* DNA. The same results were obtained using *C. salmositica* DNA and CHSE 214 cell line DNA. In both cases, as little as 1 pg (in a 15-μl PCR) of parasite DNA was detected in the absence of cell line DNA. However, in the presence of cell line DNA (15 ng in a 15-μl PCR), at least 60 pg of parasite DNA was required for detection by UNonMet-PCR. However when *T. danilewskyi* DNA was mixed with *C. auratus* DNA, the UNonMet-PCR detected *T. danilewskyi* DNA when the concentration of *C. auratus* DNA was not greater than 1,000× that of *T. danilewskyi* DNA (Fig. 3). In this case, 10 pg of *T. danilewskyi* DNA was detected in 10 ng (in a 15-μl PCR) of *C. auratus* DNA.

Assay of natural parasitic infections. Histopathological examinations indicated that the intensity of natural *Hematodinium* sp. infections varied among *C. tanneri*. The parasite was detected in 13 of 67 *C. tanneri* via histopathology and samples from all 13 positive crabs produced a strong 450-bp band with the *Hematodinium*-specific PCR assay. The UNonMet-PCR produced a single strong 600-bp band in all samples from the 13 infected *C. tanneri*. The UNonMet-PCR products from two of the 13 samples were sequenced and both were found to be 97% identical to the *Hematodinium* sp. sequence provided in GenBank (Table 2). In the remaining 54 samples two or more bands ranging from 400 bp to 600 bp were produced by the UNonMet-PCR. Of these, 19 *C. tanneri* had faint 450-bp bands with the *Hematodinium*-specific PCR probably indicating that light infections (not detectable by histopathology) were present.

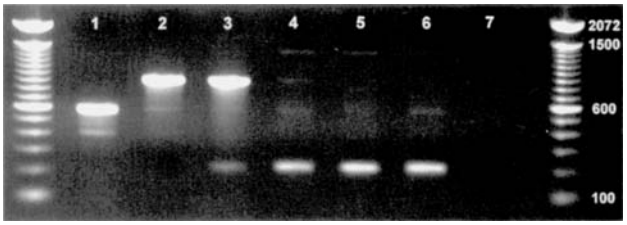

Fig. 3. Amplification of protistan (*Trypanosoma danilewskyi*) DNA in mixtures with metazoan (*Carassius auratus*) DNA by UNonMet-PCR as visualised with ultraviolet light in agarose gel (1.5%) stained with ethidium bromide. The first and last lanes are molecular markers, lane 1 contains DNA from the positive control (*Alexandrium catenella*), and lane 7 contains the negative control (ddH_20). Lane 2 contains only *T. danilewskyi* DNA (10 ng in the 15-µl reaction). Lane 3 contains equal amounts of *T. danilewskyi* and *C. auratus* DNA (10 ng of each in the 15-µl reaction). Lane 4 contains 1,000× more *C. auratus* DNA than *T. danilewskyi* DNA (dilution end point for the detection of the protistian DNA with 10 ng of *C. auratus* DNA and 0.01 ng of *T. danilewskyi* DNA in the 15-µl reaction). Lane 5 contains 10,000× more *C. auratus* DNA than *T. danilewskyi* DNA (10 ng of *C. auratus* DNA and 0.001 ng of *T. danilewskyi* DNA in the 15-µl reaction). Lane 6 contains only *C. auratus* DNA (10 ng in the 15-µl reaction). The annealing temperature for all reactions was 50 °C.

DISCUSSION

Characterization of the SSU rDNA of "new" or cryptic protistan parasites by PCR is difficult because "universal" eukaryotic PCR primers amplify host DNA as well as parasite DNA. Host DNA can predominate in bulk mixtures and under these conditions may be amplified to the exclusion of the parasite DNA. To determine whether the PCR described here was protistan-specific, we surveyed the UNonMet-PCR target sequences of 294 metazoan, protistan, bacterial, and archaeal organisms from GenBank. The identity of the surveyed species and accession numbers are available upon request. Conservation of the target site for primer 18S-Euk581-F was very high across all taxa, with only positions 4 (a T instead of a C) and 9 (a C instead of an A) in most Archaea being exceptions (Fig. 1). The target site for 18S-Euk1134-R was highly conserved in the region corresponding to the 5′ end of this 19-bp anti-sense primer and weakly conserved at positions corresponding to bases 12 and 13 in the primer. The region corresponding to the 3′ end of the primer contained distinct metazoan and non-metazoan motifs. The sequence CGCAAG (corresponding to primer nucleotides 14 to 19) was strongly conserved across all *except* the metazoan taxa. Among the metazoa, TGCAAA was the homologous motif. There were exceptions: all surveyed ctenophores and poriferans carried the non-metazoan motif, CGCAAG, as did the myxozoa, which may be derived cnidarians (Siddall et al. 1995) or of bilaterian ancestry (Anderson, Canning, and Okamura 1998). Subsequent BLAST searches of the GenBank database revealed that some representatives of many protistan taxa (e.g. Alveolata, Entamoebidae, Paramyxea, Polycystinea, Rhodophyta, and stramenopiles) possessed the metazoan target motif, TGCAAA (or the intermediate TGCAAG), and so might not amplify with these primers. Therefore, this PCR is not strictly protist-specific. It might, however, be expected to preferentially amplify many symbiotic or parasitic micro-organisms—including protists, Bacteria, Archaea, Fungi, and Myxozoa—from most metazoan hosts.

Although the UNonMet-PCR was theoretically predicted to amplify a 400-bp segment of the 16S rDNA gene in bacteria, the application of this PCR did not function as predicted for at least two Gram positive and two Gram negative bacteria. The preferential production of significantly larger amplicons in all four species of bacteria assayed indicated that the UNonMet-PCR amplified other regions of the genome. Further investigations will be required to optimise and validate the procedure for effectively amplifying DNA of bacterial pathogens in infected metazoans using the UNonMet-PCR. However, primers designed for amplifying the 16S rDNA of bacteria (e.g. Jackson et al. 2001; Rainey et al. 1992) may be better suited for the isolation of pathogen DNA from bacterial infections.

Our validation of the UNonMet-PCR revealed three caveats that must be taken into consideration when using this tool to amplify a segment of the SSU rDNA of parasitic micro-organism from infected metazoa. Firstly, organisms such as ubiquitous fungi and bacteria that can occur as contaminants in samples could produce misleading results because segments of their SSU rDNA are amplifiable by the UNonMet-PCR. Examples of contaminants that were encountered during this study include: 1) some of the multiple and usually faint (background) bands obtained in some of the invertebrate samples (e.g. *C. gigas*, *C. tanneri* and *C. magister*), believed to be free of significant protistan infections, may have been derived from cryptic symbionts or parasites that contaminated the samples (e.g. a clone of the bands produced by UNonMet-PCR of one *C. gigas* resulted in the isolation of DNA most similar to *Ephelota* sp., a species of suctorian ciliate); 2) the difference in the banding pattern between the fish cell lines (EPC and CHSE 214) and fish tissue (*C. auratus* and *O. tshawytscha*) suggested the presence of a contaminant in the cell lines which was subsequently identified as a *Bacillus* sp. (Robinson, J., pers. commun.); 3) the parasite *Loma salmonae* was harvested from the gills of laboratory infected fish by Percoll gradient centrifugation, a process that could also enrich for gill contaminants such as yeast as indicated in Table 2 (SRMJ., pers. commun.); and 4) the DNA sample for the fungus *Cronartium ribicola* had been extracted from aecia and aeciospore tissue contained within the blister that forms on the stems and branches of infected western white pine trees (*Pinus monticola*). The fungus cannot be completely separated from tree tissues therefore the sample was expected to be contaminated with a small amount of host DNA (Piggott, N., pers. commun.). In this case, the UNonMet-PCR amplified the plant DNA instead of the fungus. Further assessment will be required to determine the applicability of the UNonMet-PCR to parasites of plants. Because the UNonMet-PCR can target sequences of secondary contaminants in metazoan samples, care must be taken to avoid contamination and thus amplification of contaminant DNA.

The second caveat is the propensity of the UNonMet-PCR to inconsistently amplify metazoan DNA in the absence of non-metazoan DNA. Although the bands produced from all metazoa that were tested tended to be faint, numerous, ranging in size from about 200–2,000 bp, and inconsistently produced at various annealing temperatures, a single band of about 600 bp was obtained from some samples. The sequence produced by the 600-bp band amplified from samples of two Dungeness crabs (*C. magister*) showed a 97% homology to the SSU rDNA of the calico box crab (*H. epheliticus*), demonstrating that crustacean DNA was amplified (no sequence for the SSU rDNA of *C. magister* occurs in Genbank). The sequence from bands produced by the three other metazoa also matched that of corresponding species listed in GenBank (see Table 2). However, when sufficient non-metazoan DNA (i.e. the parasite of interest) was present in the sample, the non-metazoan DNA was amplified in preference to that of the metazoan DNA.

The third caveat is the importance of having a sufficient amount of non-metazoan (pathogen) DNA present in the sample containing metazoan (host) DNA in order for the UNonMet-PCR to successfully amplify the non-metazoan DNA. By using

mixtures of parasite DNA and DNA from host cell lines (i.e. *T. danilewskyi* DNA with EPC (*C. carpio*) cell line DNA and *C. salmositica* DNA with CHSE-214 (*O. tshawytscha*) cell line DNA), we determined that a band corresponding to amplification of parasite DNA was produced when the concentration of the cell line DNA did not exceed approximately 250× that of the parasite DNA. However, *T. danilewskyi* DNA was detected by UNonMet-PCR in mixtures with four times as much DNA obtained directly from the goldfish host. The presence of a contaminating *Bacillus* sp. in the EPC cell line possibly provided competition for the UNonMet primers thereby reaffirming the first caveat and exemplifying the impact that contaminants may have on results. Alternately, DNA for *C. carpio* and *C. auratus* may be differentially amplified by the UNonMet-PCR. Examination of crabs naturally infected with *Hematodinium* provided further support for the third caveat. Amplification of DNA from heavily infected *C. tanneri* resulted in a strong 600-bp band identified as *Hematodinium*, in contrast to the faint or multiple bands produced from lightly infected or non-infected crabs.

In conjunction with the three caveats described above, it is necessary to have appropriate uninfected controls when using this primer set in order to characterize bands that may be produced by non-specific amplification of metazoan DNA. In addition, characterization of the PCR product(s) by techniques such as sequencing or restriction analysis is essential in confirming the identity of the amplicon(s). Nevertheless, during our validation process, the UNonMet-PCR proved useful in specifically identifying two phytoplankton cultures (*Isochrysis* sp. (CCMP 1324) as *Isochrysis galbana* and *Chrysochromulina* sp. as *Chrysochromulina hirta*) and indicated the contamination of another phytoplankton culture (the isolate thought to be *Rhodomonas* sp. was a species of *Tetraselmis* sp.). In addition, partial sequences of the SSU rDNA of five organisms (*Cancer magister*, *Trypanosoma danilewskyi*, *Pinus monticola*, *Chaetoceros gracilis*, and *Chionoecetes tanneri*) not previously available in GenBank were added to the database (accession numbers AY527220, AY527221, AY527222, AY527223, AY527224, respectively). Future research could include the characterisation of secondary bands obtained in many of the samples. In addition, further refinement of the UNonMet-PCR could reduce the production of the secondary bands and increase the specificity of the primer set.

The application of PCR using the UNonMet-PCR could be a significant research tool as new fungal and protistan parasitic diseases of metazoa emerge. The taxonomic affinities of a new or cryptic parasite are normally deduced only after expensive morphological analysis often including time-consuming ultrastructural examinations by electron microscopy. Genetic characterisation may lag behind because of problems associated with isolating the organism or amplifying its DNA from host tissue. Using this PCR assay, parasite SSU rDNA could be rapidly amplified for sequencing and its phylogenetic affiliation deduced within days. Inferences about a potential parasite life cycle could follow based on the life histories of other members of its taxon. Thus, this non-metazoan PCR assay might expedite the description of new species and emerging pathogens.

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